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Anti-chlamydial dimeric indole derivatives from marine actinomycete *Rubrobacter radiotolerans*

Jian Lin Li[1,*], **Dandan Chen**[1,2,*], **Lei Huang**[1], **Min Ni**[1], **Yu Zhao**[1], **Huizhou Fan**[3], **Xiaofeng Bao**[1]

[1]School of Pharmacy, Nantong University, Nantong, China

[2]Department of Pharmacy, The Second People's Hospital of Nantong, Nantong, China

[3]Robert Wood Johnson Medical School, Rutgers University, Piscataway, New Jersey, USA

Abstract

Chlamydiae are widely distributed pathogens of human populations, which can lead to serious reproductive and other health problems. In our search for novel antichlamydial metabolites from marine derived microorganisms, one new (**1**), along with two known (**2**, **3**) dimeric indole derivatives were isolated from sponge derived actinomycete *Rubrobacter radiotolerans*. The chemical structures of these metabolites were elucidated by NMR spectroscopic data as well as CD calculations. All three metabolites suppressed chlamydial growth in a concentration-dependent manner. Among them, compound **1** exhibited the most effective antichlamydial activity with IC_{50} values of 46.6 ~ 96.4 μM in production of infectious progeny. Compounds appeared to target the mid-stage of the chlamydial developmental cycle by interfering with reticular body replication but not directly inactivating infectious elementary body.

Keywords

Chlamydia; dimeric indole; sponges; actinomycete; *Rubrobacter radiotolerans*; antichlamydial

Introduction

Chlamydiae are a group of obligate intracellular bacterial pathogens responsible for diseases in a range of hosts including humans [1, 2]. *Chlamydia trachomatis* is arguably the most common cause of sexually transmitted infections worldwide. Urogenital chlamydial infections frequently lead to serious complications including ectopic pregnancy, abortion, infertility, and pelvic inflammatory disease. In addition, *C. trachomatis* infection is the leading cause of eye disease as well, causing blindness in approximately 6 million and

Correspondence: Prof. Xiaofeng Bao, School of Pharmacy, Nantong University, 19 Qixiu Road, Nantong 226001, Jiangsu, China, Phone: +86 513 85051859, Fax: +86 513 85051858, baoxi@ntu.edu.cn.

*These two authors contributed equally to this work.

Supporting information

^{1}H, ^{13}C and 2D NMR data of compound **1** and concentration-response curves for generating IC_{50} values of three compounds on each *Chlamydia* strain are available as Supporting Information.

Conflict of Interest

The authors declare no conflict of interest.

affecting the health of over 400 million worldwide [3, 4]. *Chlamydia pneumoniae* is an etiologic agent of respiratory diseases such as pneumonia and bronchitis and a possible risk factor for atherosclerosis, stroke and Alzheimer's disease [5–7].

Chlamydiae have a unique biphasic life cycle consisting of two alternating cellular forms, the infectious but non-proliferative elementary body (EB) and the replicative but non-infectious reticulate body (RB). Chlamydial infection is initiated by EB adhering to and invading the host cell, and forming a specialized vacuole, the so-called inclusion. Within the inclusion, EB differentiates into RB. After round of replication, RBs reorganize back to EBs. Most chlamydial developmental cycles are complete in 40–72 hours when EBs are released from the host cell [1, 8–10].

Current therapies of chlamydial infections are broad-spectrum antibiotics, including azithromycin, doxycycline and fluoroquinolones [11–13]. Although chlamydiae are susceptible to a number of antibiotics, data suggest that incomplete antibiotic therapy can result in persistence and long-term infection [14]. In addition, antibiotic-resistant chlamydiae have been detected [11, 15–18]. During the past few decades, plenty of *Chlamydia* research efforts have been focused on vaccine development, but not resulting in an effective vaccine for human use [19, 20]. The merge of antibiotic-resistant chlamydiae and lack of approved vaccine necessitates identification of novel antichlamydials through chemical synthesis [21–23] or from natural resources [24–26].

Endozoic microorganisms inhabiting the inner tissues of animals produce of a huge diversity of secondary metabolites that deliver interesting pharmacological properties and are recognized as a prolific source of biologically active molecules [27]. In our continuous search for antichlamydial metabolites from marine derived microorganisms, we generated one new (**1**), along with two previously reported dimeric indole derivatives (**2**, **3**) [28] from sponge derived actinomycete *Rubrobacter radiotolerans*. We found that all the three metabolites inhibited *Chlamydia* in a concentration-dependent manner, with compound **1** being most effective.

Results and Discussion

Compound **1** was obtained as a yellow, amorphous solid and was found to have a molecular formula of $C_{21}H_{22}N_2O_3$ by HRESIMS analysis. Absorption observed at 3445 cm^{-1} in its IR spectrum suggested the presence of hydroxyl functionality. Additionally, sixteen olefinic carbons at δ_C 138.3–108.5 and three oxygen-bearing carbons at δ_C 74.3–61.9 were observed. The remaining carbons were also categorized by the DEPT spectrum.

The NMR data of compound **1** was almost identical to that of compound **2** [28]. However, in HPLC analyses, two distinct peaks were generated. In combination with nuances of ^{13}C NMR data of the side chain (C-8′–C-10′), compounds **1** and **2** were suggested to be epimer. This result was further confirmed by using optical rotation value and CD analysis, which showed a positive cotton effect (CE) around 223 nm and a negative CE around 216 nm of compound **1**, suggested the absolute configuration of C-8′ was unambiguously as 8′*R* (Fig. 1).

We hypothesized that all the three compounds were derived from condensation of two tryptophan molecules. The early steps of the biosynthetic process were proposed to involve the deamination of a tryptophan unit to yield 3-(1*H*-indol-3-yl)acrylic acid. The newly formed allyl part attacked another tryptophan moiety at position 2 to obtain intermediate material, resulting in the formation of both *R* and *S* isomers in our study. And then, the intermediate material was further underwent oxidation-reduction to give compounds **1** to **3** (Fig. 2).

*Compound **1**:* yellow powder; $[\alpha]^{23}_{D}$ −8.9 (*c* 0.05, MeOH); UV (MeOH) λmax (log ε) 203 (2.54), 225 (2.16), 278 (1.94) nm; IR v_{max} 3445, 2927, 2856, 1408, and 1383 cm^{-1}; CD (*c* 1.4×10^{-3} M, MeOH) Δϵ (nm) 4.92 (212); −9.46 (226); ^{1}H-NMR (CD_3OD): 7.51 (1H, dd, *J* = 7.8, 1.0, H-4′); 7.45 (1H, dd, *J* = 7.8, 1.0, H-7′); 7.44 (1H, s, H-2′); 7.30 (1H, dd, *J* = 8.1, 1.0, H-4); 7.26 (1H, dd, *J* = 8.0, 1.0, H-7); 7.03 (1H, ddd, *J* = 8.0, 7.8, 1.0, H-6′); 6.99(1H, ddd, *J* = 8.0, 7.8, 1.0, H-5′); 6.93 (1H, ddd, *J* = 8.1, 8.0, 1.0, H-5); 6.93(1H, ddd, *J* = 8.0, 8.0, 1.0, H-6); 4.78 (1H, d, *J* = 5.7, H-8'); 4.40 (1H, ddd, *J* = 10.5, 5.7, 4.0, H-9′); 3.70 (2H, t, *J* = 7.0, H-9); 3.57 (1H, dd, *J* = 10.5, 4.0, H-10′); 3.45 (1H, dd, *J* = 10.5, 5.7, H-10′); 3.08 (2H, t, *J* = 7.0, H-8); 138.3 (C-2); 137.7 (C-7a′); 137.3 (C-7a); 129.1 (C-3a′); 127.5 (C-3a); 122.8 (C-6′); 122.4 (C-2′); 121.1 (C-5′); 119.4 (C-4′); 119.4 (C-6); 119.1 (C-5); V118.0 (C-7′); 114.7 (C-3′); 110.7 (C-4); 110.4 (C-7); 108.5 (C-3); 74.3 (C-9′); 65.5 (C-10′); 61.9 (C-9); 36.9 (C-8′); 29.1 (C-8); (+)-ESIMS *m/z* 373 $[M + Na]^{+}$.

The antichlamydial effect of compounds **1**–**3** was first determined using *C. trachomatis* L2, a sexually transmitted serotype responsible for lymphogranuloma venereum. Compared with negative control DMSO (final concentration, 0.5%), compounds **1**–**3** all inhibited both formation and growth of *C. trachomatis* L2 inclusions in a concentration-dependent manner. Compound **1** was the most effective, which completely blocked inclusion formation at 480 μM (Fig. 3A), analogous to the effect of positive control tetracycline (final concentration, 11.25 μM) [26]. Yields of infectious progeny EBs were further determined to evaluate the inhibitory effect of compounds **1–3**. As expected, all three compounds exhibited similar concentration-dependent inhibition and 480 μM of compound **1** or positive control completely inhibited the production of progeny EBs (Fig. 3B). Using non-linear regression analysis by Graphpad Prism 5 software, the IC_{50} values, defined as the concentration at which the yield of infectious progeny EBs was reduced by 50% relative to negative control and presented as Mean (95% confidence interval), were calculated as 85.62 μM (77.45 ~ 94.65 μM), 227.0 μM (198.1 ~ 260.0 μM) and 238.7 μM (209.8 ~ 271.7 μM) for compounds **1**–**3**, respectively.

As *Chlamydia* relied on the host cells for survival and replication, it was important to confirm whether the observed inhibitory effect was due to the direct action of the compounds on *Chlamydia* rather than an indirect cytotoxic effect on the host cells. Potential cytotoxic effect of compounds **1–3** on host cells was assessed by treatment of uninfected cells with 480 μM of individual compound. 48 hours later, no substantial cytotoxic effect on host cells was observed from cell proliferation by counting cell numbers, morphological feature by Evan's blue staining and nuclear integrity by DAPI staining (Fig. 4), indicating that compounds **1–3** were well tolerated by HeLa cells and their inhibitory effect was due to the direct action on *Chlamydi*a but not cytotoxicity.

The antichlamydial effect of compounds **1**–**3** was additionally confirmed using *C. trachomatis* C, a blinding ocular serotype, *C. trachomatis* D, one of the most common sexually transmitted serotype, and *C. muridarum* strain Nigg II, a mouse pathogen used to model human chlamydial infections in mice by evaluating the yields of infectious progeny EBs. All three *Chlamydia* strains were inhibited by compounds in similar concentration-dependent pattern as observed in *C. trachomatis* L2, only with slight differences of susceptibility (Fig. 5). As expected, compound **1** was the most active one, which fully abolished the production of progeny EBs at 480 μM, analogous to positive control tetracycline. The IC_{50} values of the three compounds on all *Chlamydia* strains were calculated based on concentration-response inhibition data by generating concentration-response curves (Figure 5S) and presented in Table 1.

Chlamydia is characterized by its unique developmental cycle of EB/RB alternation and this distinctive lifecycle offers multiple opportunities for therapeutic intervention. To understand whether compounds **1–3** exerted their antichlamydial effect by affecting the infectivity of EBs and/or the growth of RBs, *C. trachomatis* L2 EBs were pretreated with 480 μM of compounds **1–3** or 0.5% DMSO for 1 h at 4°C, and their titers were determined on HeLa cell monolayers after washing off the compounds. No detectable change of inclusion counts was observed (Fig. 6), suggesting that compounds **1**–**3** did not directly inactivate EBs. Next, we added individual compound to infected cultures at different times postinoculation (Fig. 7A), and then quantified infectious progenies that formed in these cultures. We found addition of 240 μM compound **1** at 0, 2 and 12 h postinoculation resulted in approximately 95% inhibition (Fig. 7B), whereas inhibition efficiency significantly dropped to 85% when the inhibitor was added at 24 h. Compounds **2** and **3** postinoculation revealed similar inhibition pattern (Fig. 7B). Since the EB differentiates into the RB around 6 h after cell entry, and RBs then replicate exponentially before they asynchronously reorganize into EBs [1], the time point inhibition data indicated that compounds **1**–**3** specifically targeted the mid-stage of the chlamydial developmental cycle by interfering with RB replication. Future studies are needed to identify detailed inhibition mechanism.

In conclusion, one new dimeric indole metabolite, compound **1**, along with the previously known metabolites (**2**, **3)** were isolated and identified from the sponge's endozoic actinomycete *Rubrobacter radiotolerans*. All three metabolites suppressed chlamydial growth in a concentration-dependent manner, which targeted the mid-stage of the chlamydial replication cycle. Among them, compound **1** was the most effective one. Our findings highlighted the potential of marine derived microorganisms as sources for screening promising lead molecules for development of antichlamydial agents.

Materials and Methods

Biological material isolation and identification

The actinomycete strain was isolated from a marine sponge *Petrosia sp.*, which was collected by scuba (15 to 25 meters depth) in 2013, off the coast of Xisha Islands, China. Following a rinse with sterile sea water, small pieces of the surface and inner tissue of the sponge were homogenized and then inoculated. The pure actinomycete strain, designated as

05039, was identified as *Rubrobacter radiotolerans* by a morphological and biochemical analysis by Professor Guangtong Chen. A voucher sample (No PF2159) was deposited at the Department of Pharmacy, Nantong University.

Extraction, purification and structure elucidation

Fermentation was performed in 100 mL malt media in 300 mL Erlenmeyer flasks for subculture. For the massive culture, 100 mL of subculture was transferred into a 1 L Erlenmeyer flask containing 0.5 L culture media and fermentation was carried out on a rotary shaker (30°C, 150 rpm, 10 days). The cultured actinomycete (20 L) was extracted with 10 L of EtOAc, to afford the EtOAc extract, which was partitioned between n-hexane and 90% MeOH. The 90% MeOH layer was subjected to a stepped-gradient MPLC eluting with 30% to 100% MeOH to afford 16 fractions. Fraction 6 (50% MeOH fraction) was subjected to RP-HPLC (YMC packed J'sphere ODS-H80 column, 250 × 10 mm, 4 μm, 80 Å), eluted with 35% (1.0 mL/min, 220 nm) aqueous MeOH to afford compounds **2** (2.0 mg, Rt 90 mins) and **3** (1.4 mg, Rt 55 mins). Compound **1** was isolated from fraction 7 (55% MeOH fraction) with 35% (1.0 mL/min, 220 nm) aqueous MeOH (2.3 mg, Rt 62 mins) and further purified by sephadex LH-20 column, eluted with MeOH and CH_2Cl_2 (1:1).

Chlamydial strains and culture conditions

C. trachomatis C (strain TW3), D (strain UW-3/CX) and L2 (strain 434/Bu) and *C. muridarum* (strain Nigg II) were purchased from ATCC. HeLa cells purchased from ATCC were used for all cell culture experiments, and were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich) and 10 μg/mL gentamicin at 37°C in a humidified atmosphere of 5% CO_2. EB stocks were prepared in sucrose-phosphate-glutamate (SPG) and stored at −80°C as previously described [21].

Chlamydia infection and inhibition assay

The inhibition effect of compounds was evaluated as previously described, and quantified by IC_{50} [21, 26]. Cell monolayers were seeded onto coverslips in 24-well plate for immunofluorescence staining or directly in 48-well plate for determining the production of infectious progeny EBs. After overnight incubation, cells with 60~70% confluence were infected at multiplicity of infection (MOI) 0.2 inclusion-forming unit (IFU) per cell with different chlamydial strains. Individual compound, dissolved in DMSO, was added to final concentrations of 30, 60, 120, 240, or 480 μM simultaneously with chlamydial inoculation. DMSO (final concentration, 0.5%) or 11.25 μM tetracycline (Sigma Aldrich, purity 99%) [26] was used as negative or positive controls. Centrifugation (900 × g for 1 h at room temperature) was used to facilitate infection of the C. *trachomatis* C and D serotypes. Thirty-six (C. *trachomatis* L2 and *C. muridarum*) or 40 hours (C. *trachomatis* C and D) postinfection, cells were scraped or fixed with prechilled methanol at room temperature for 10 min. Methanol-fixed cells were kept in PBS at 4°C until subjected to sequential staining processed with a primary antibody and a FITC-conjugated secondary antibody. Evans blue was used as counterstain. Images were shot by Olympus IX51 fluorescence microscope. The scraped-off cells were disrupted by brief sonication to release infectious progeny EBs. The

lysates were used to infect HeLa cell monolayers grown in 96-well plate following 1:10 serial dilution. The recoverable IFUs were quantified by immunofluorescence staining described as above. Percent inhibition was calculated based on recoverable IFUs in compound-treated samples relative to untreated negative control. IC_{50} values were calculated by non-linear regression from the concentration-response inhibition data using Graphpad Prism 5 software.

Cytotoxicity of compounds toward HeLa cells

HeLa cells were seeded onto 24-well plate at a density of 2×10^4 cells per well. After overnight growth, cells were switched into culture medium containing 480 μM of individual compound or 0.5% DMSO. Forty-eight hours later, cells were fixed with methanol and stained with Evans blue for morphology or DAPI for nuclear integrity. Otherwise, 48 hours later, cells were detached from the plastic by trypsinization and enumerated by hemacytometer.

Elementary body infectivity assay

L2 EBs were suspended into SPG at 4×10^5 IFU/mL and exposed to 480 μM of individual compound or 0.5% DMSO for 1 h at 4°C. After twice washing with SPG to eliminate the residual chemical, EBs were suspended into culture medium and immediately added to HeLa cell monolayers. Thirty-six hours later, infective EBs counts were determined by measuring inclusion counts after immunofluorescence staining as above.

Statistical analysis

All experiments were carried out as three replicates in minimum and results except IC_{50} were expressed as mean ± standard deviation. IC_{50} values, presented as Mean (95% confidence interval), were calculated by non-linear regression choosing “log(inhibitor) vs. normalized response – Variable slope” equation from the concentration-response inhibition data using Graphpad Prism 5 software. For statistical analysis, Student’s t-test ($p < 0.05$ or $p < 0.01$) was applied.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

EB	Elementary body
RB	Reticular body

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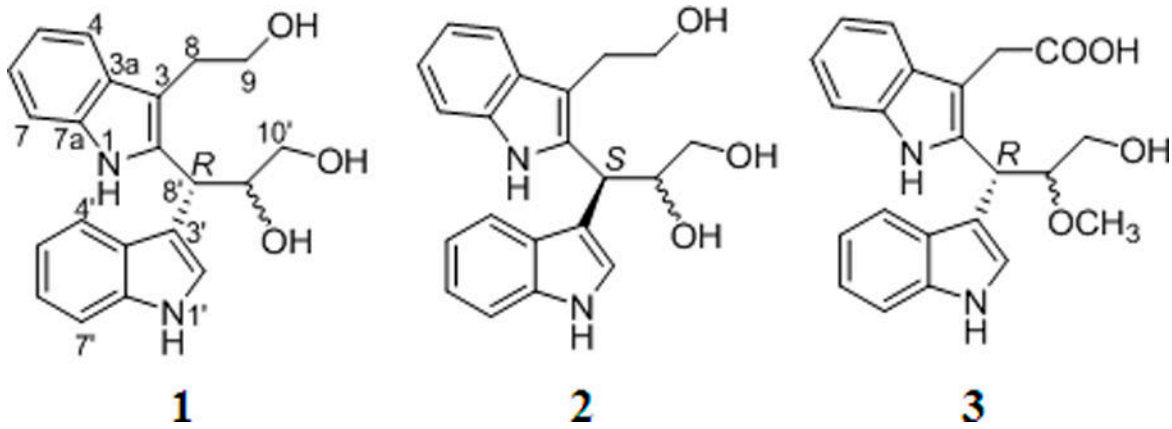

Fig. 1.
Chemical structures of compounds **1**–**3**.

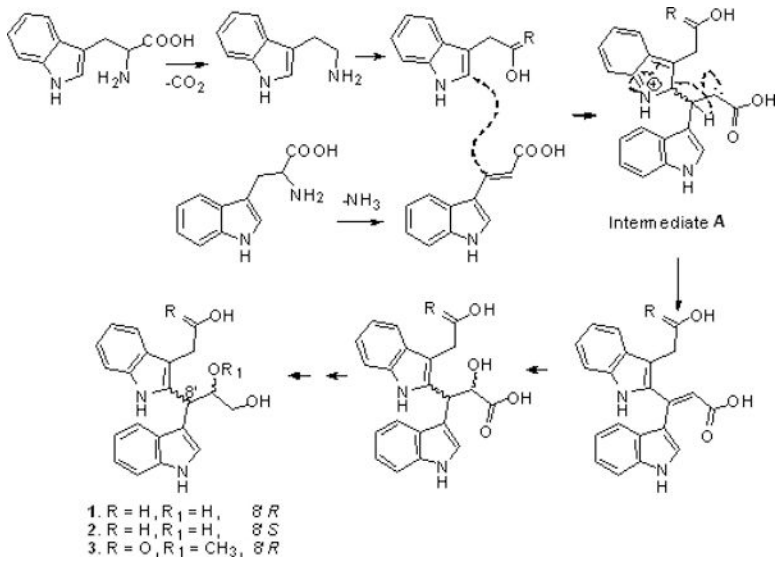

Fig. 2.
Plausible biosynthetic pathway of compounds **1**–**3**.

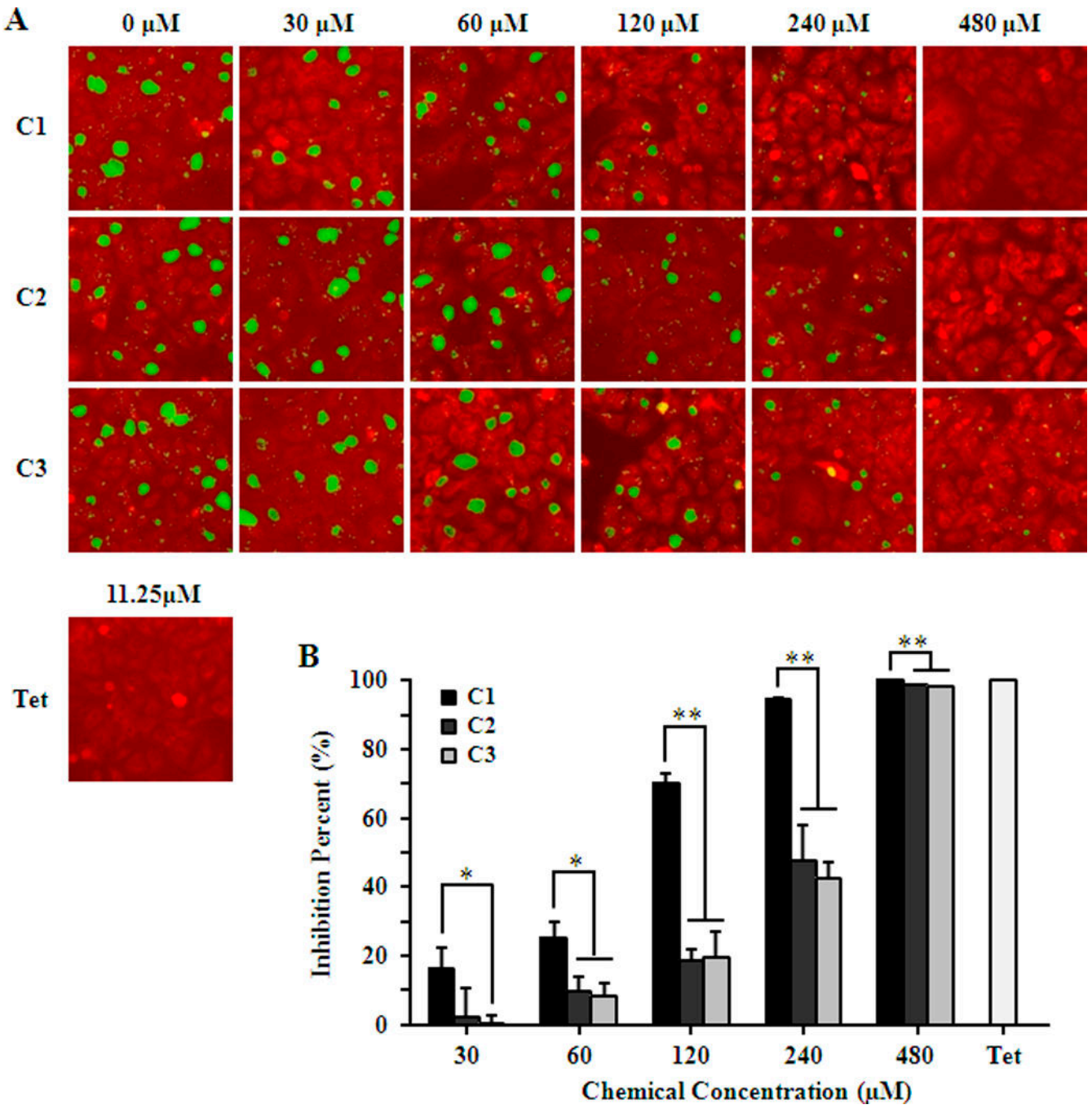

Fig. 3.

Inhibition effect of compounds on *C. trachomatis* L2. HeLa cells were infected with *C. trachomatis* L2 EBs at a MOI of 0.2 in the presence of 0.5% DMSO, 11.25 μM tetracycline (Tet) or various concentrations of compounds **1**–**3**. (A) Cells were fixed 36 hours post-infection. The chlamydial inclusions were strained with a polyclonal anti-EB antibody (green) and cells were counterstained with Evans blue (red). (B) The inhibitory effect of compounds on the production of infectious progeny EBs was determined by counting recoverable IFUs in compound or Tet treated samples relative to untreated negative control.

Asterisks indicate compound **1** inhibition levels were significantly different from compound **2** and **3** (*P<0.05, **P<0.01 by Student's t test). (Color figure available online only)

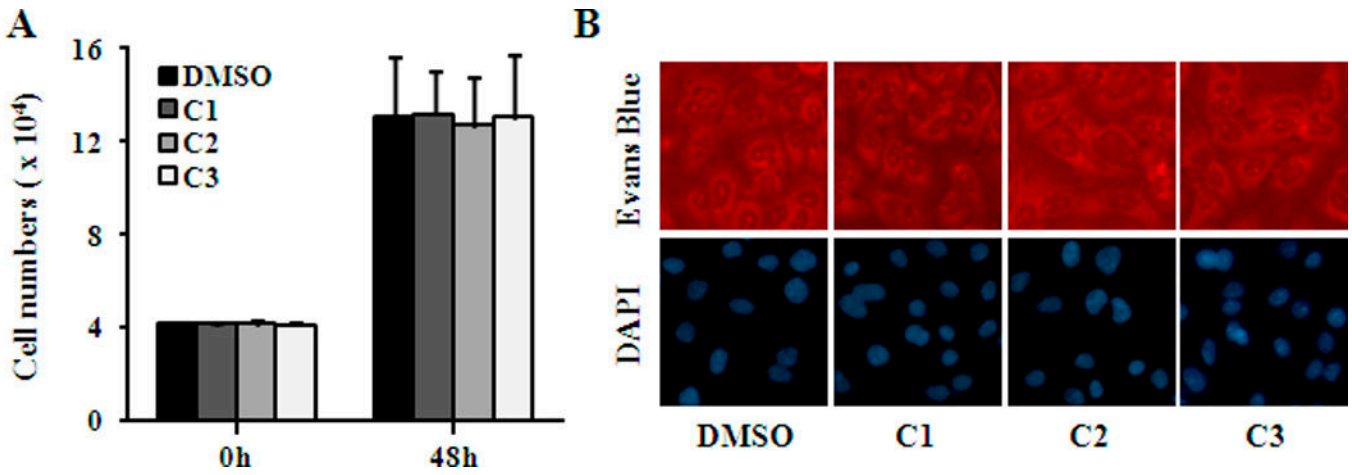

Fig. 4.

Lack of toxicity of compounds to host cells. Uninfected HeLa cells at 20% confluence were cultured with medium containing 480 μM individual compound or 0.5% DMSO and were enumerated (A) or stained with Evans blue or DAPI (B) 48 h later. (Color figure available online only)

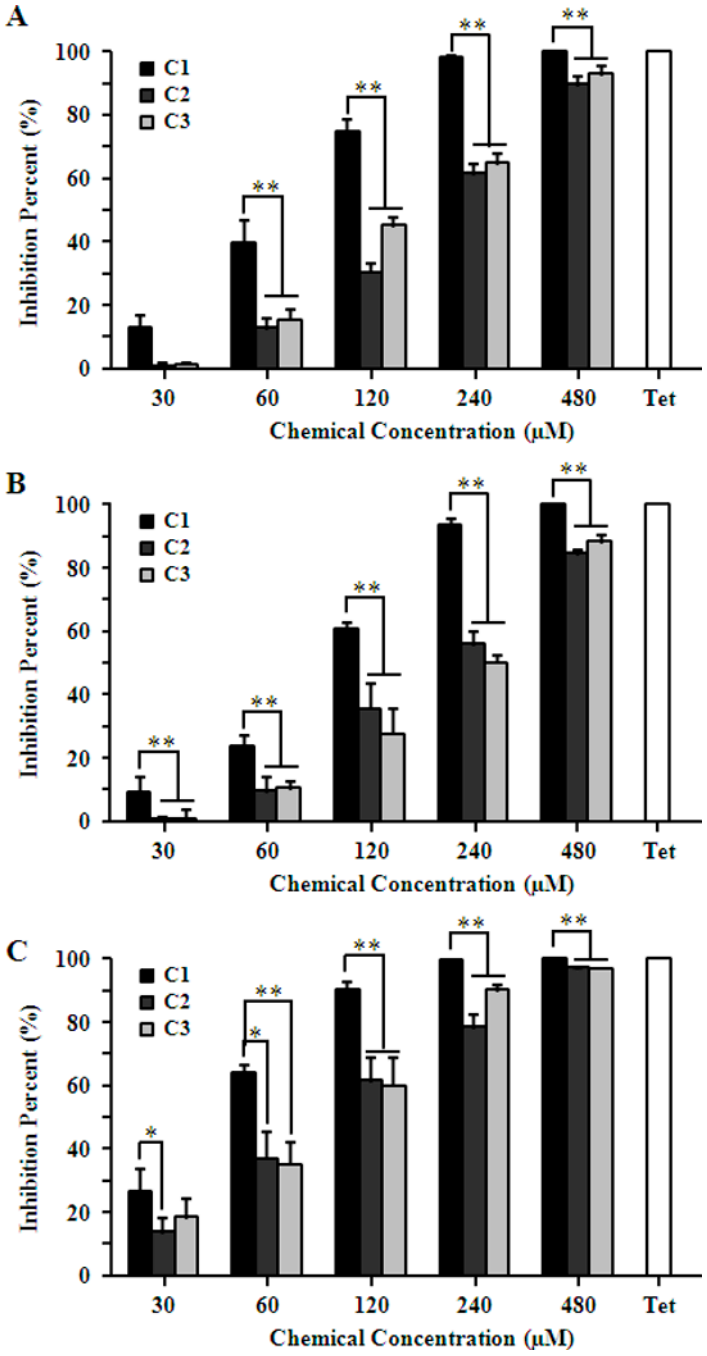

Fig. 5.

Inhibition effect of compounds on *C. trachomatis* C and D serotypes and *C. muridarum*. HeLa cells were infected with *C. trachomatis* C (A) or D (B) or *C. muridarum* (C) EBs at a MOI of 0.2 in the presence of 0.5% DMSO, 11.25 μM tetracycline (Tet) or various concentrations of compounds **1**–**3**. The inhibitory effect of compounds on the production of infectious progeny EBs was determined by counting recoverable IFUs in compound or Tet treated samples relative to untreated negative control. Asterisks indicate compound **1**

inhibition levels were significantly different from compound **2** and **3** (*P<0.05, **P<0.01 by Student's t test).

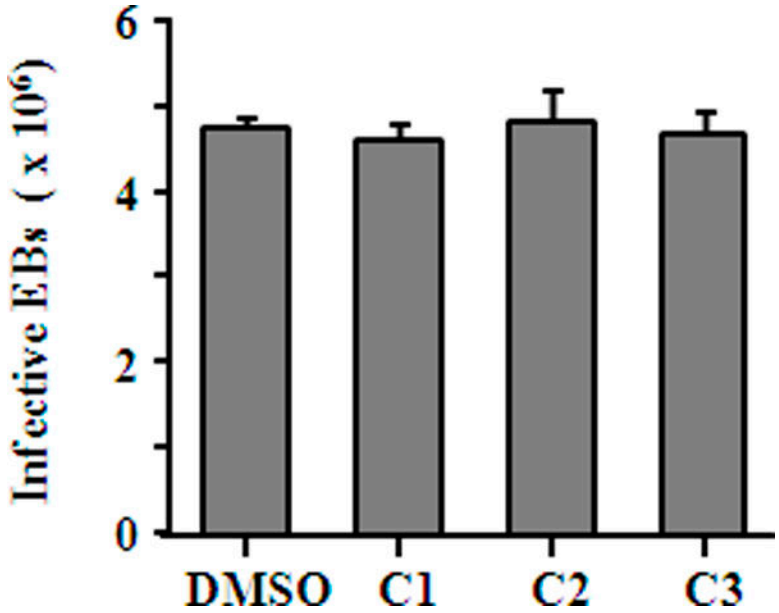

Fig. 6.

Lack of direct effect of compounds on *Chlamydia* EBs. *C. trachomatis* L2 EBs were incubated with 480 μM individual compound or 0.5% DMSO at 4°C for 1 h. The residual chemical was removed from EBs by two washes with SPG. Infective EBs counts were determined by measuring inclusion counts after inoculation of HeLa cells for 36 h.

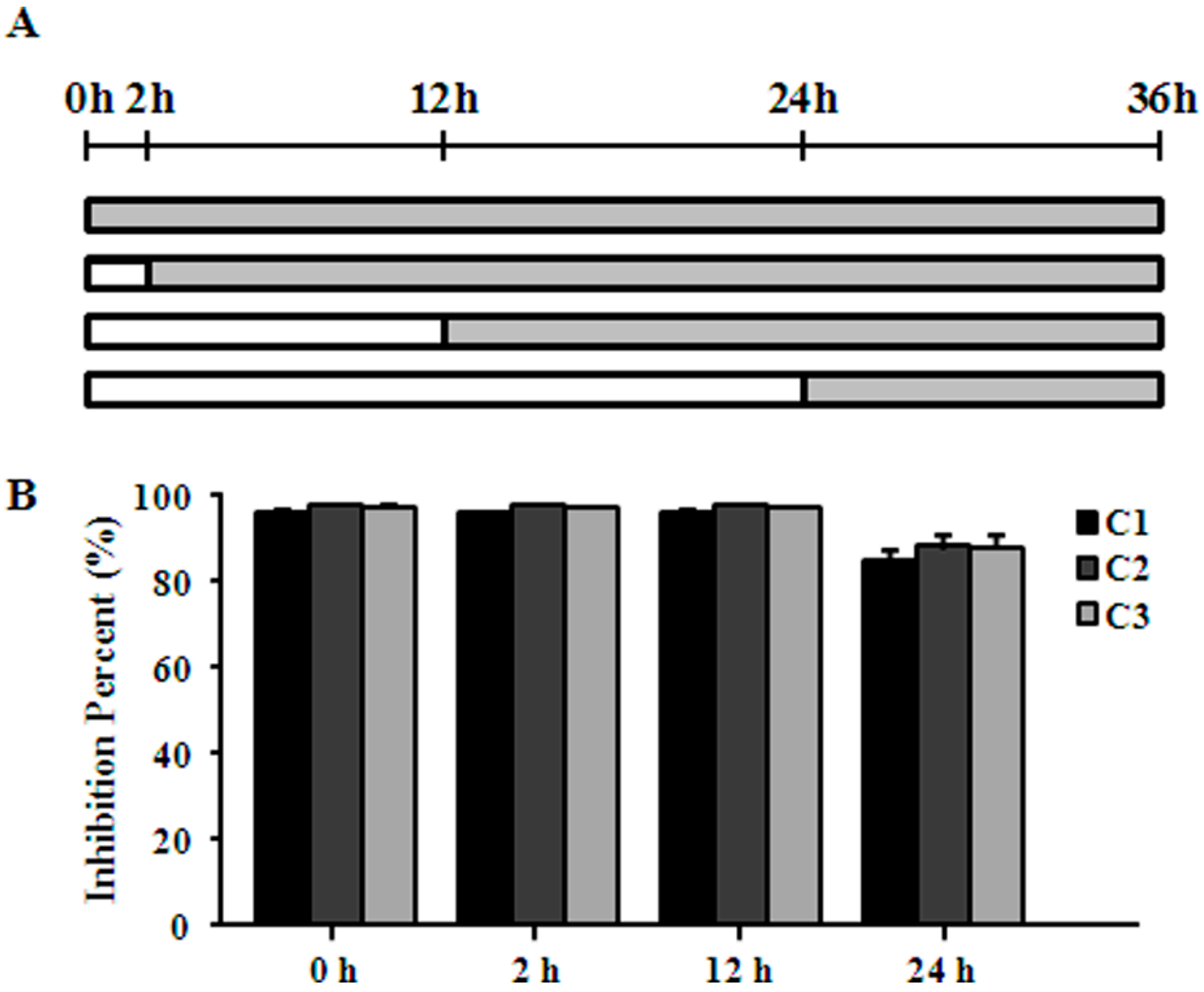

Fig. 7.

Impact of exposure times on antichlamydial effect of compounds. (A) HeLa cells infected with *C. trachomatis* L2 EBs at a MOI of 0.2 were treated with 240 μM compound **1 or** 480 μM compound **2** or **3** at different time points as described. (B) The inhibitory effect of compounds on the production of infectious progeny EBs at different time points was determined by counting recoverable IFUs in compound treated samples relative to untreated negative control at 36 h.

Table 1

IC_{50} values (μM) of compounds **1**–**3** for different *Chlamydia*. IC_{50} values were presented as Mean (95% confidence interval).

	***C. trachomatis* L2**	***C. trachomatis* C**	***C. trachomatis* D**	***C. muridarum***
C1	85.62 (77.45 ~ 94.65)	71.84 (66.76 ~ 77.30)	96.24 (90.34 ~ 102.5)	46.53 (43.97 ~ 49.24)
C2	227.0 (198.1 ~ 260.0)	181.4 (170.9 ~ 192.6)	191.7 (172.8 ~ 212.7)	89.21 (78.97 ~ 100.8)
C3	238.7 (209.8 ~ 271.7)	148.3 (133.6 ~ 164.7)	212.2 (191.3 ~ 235.5)	84.74 (74.63 ~ 96.22)